Angiotensin II Increases Cancer Stem Cell-like Phenotype in Lung Cancer Cells

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Abstract. *Background: Cancer stem cells (CSCs) have been proposed as important players in cancer progression, metastasis, and chemotherapeutic resistance in many cancers, including lung cancer. However, effects of the endogenous substance angiotensin II (ANG II) on cancer stem cell-like phenotype in lung cancer are largely unknown. Materials and Methods: Human lung cancer cells were treated with non-cytotoxic concentrations of ANG II. The CSC phenotype was evaluated by spheroid formation, 3D culture, and anchorage-independent growth assays. The expression levels of CSC makers were determined by western blot analysis. Results: ANG II significantly increased the ability of lung cancer cells to form spheroids. ANG II also increased the growth of cancer cells in a 3D culture Matrigel-based assay and facilitated cancer cell survival in an anchorage-independent condition. Western blot analysis revealed that cell treatment with ANG II significantly up-regulated CD133 levels. Conclusion: The present study revealed that the endogenous substance ANG II enhances CSC-like phenotype in lung cancer cells.*

The possible side-effects of human hormones, as well as endogenous compounds, on cancer cells have been intensively reported. Studies have indicated that the newly-identified hormone ouabain possesses anticancer, anti-migratory, and anoikis sensitization activity (1). In contrast, endogenous compounds, such as nitric oxide, have been reported for their cancer-promoting effects (2). In-depth knowledge about these endogenous species can offer a better understanding over disease and lead to the development of novel therapeutic, as well as preventive approaches. The endogenous compound angiotensin II (ANG II) has been shown to potentiate migratory activity and metastasis of breast cancer cells (3). Moreover, the role of the renin–angiotensin system (RAS) on cancer has garnered increasing attention in the cancer research field (4).

ANG II, an effector molecule in the RAS plays important roles in blood pressure regulation and cardiovascular homeostasis (Figure 1). In addition to its hemodynamic effects, ANG II is known as a growth factor contributing to cell proliferation, migration, and angiogenesis (5). Previous studies reported that different cancer cells and tissues express ANG II and RAS components (6-9), and inhibition of ANG II reduces tumor growth in various animal carcinoma models (10-14). More importantly, ANG II has been shown to promote epithelial-to-mesenchymal transition (EMT) in cancer cells, which is an important characteristic of cancer invasiveness (15-17).

Although the potentiating effect of ANG II on cancer cell aggressiveness is well-recognized, the effect of such hormone on cancer stem cell (CSC) phenotype is largely unknown. The stemness of cancer cells has been widely accepted as a critical cause of therapeutic failure, metastasis, and cancer relapse. Studying the effects of ANG II on CSC-like phenotype may lead to a further understanding over the pathology of cancer aggressiveness and progression. As more evidence has indicated the potentiating effects of such a system on cancer progression, the use of RAS inhibitors for the treatment of hypertension and cardiovascular disease such as angiotensin converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARBs) may have beneficial effects on various aspects of cancer.

In the present study we investigated the effects of ANG II on key features of cancer stem cells, including their ability to grow as 3D tumor spheroids and under anchorage-independent conditions. The expressions of CSC markers were also investigated. Knowledge obtained from this study may help highlight the possible roles of this endogenous molecule on cancer progression.

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Materials and Methods

Cell culture. Human non-small cell lung cancer cell lines, NCI-H460, were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultivated in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin (GIBCO, Grand Island, NY, USA). Cell cultures were maintained in a 37˚C humidified incubator with 5% CO_2. Cells were routinely passaged at preconfluent density using a 0.25% trypsin solution with 0.53 mM EDTA (GIBCO). Cells were seeded into 6-well plates at an initial plating density of 2×10^5 cells/well. Cells were allowed to adhere to the surface of the plates overnight, after which they were treated with different concentrations of ANG II range from 0 to 1000 μM (Sigma Chemical, St. Louis, MO, USA). The cells were subsequently collected for further analysis at indicated time points.

Cytotoxicity and proliferation assays. To evaluate cytotoxicity of ANG II, cells were seeded onto 96-well plates at a density of 1×10^4 cells/well and were allowed to incubate overnight. Cells were then treated with different concentrations of ANG II for 24 h. Cell viability was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's protocol (Sigma Chemical). For cell proliferation assay, cells were seeded onto 96-well plates at a density of 5×10^3 cells/well. Cell proliferation was then determined with MTT assay at 24, 48, and 72 h according to the manufacturer's protocol (Sigma Chemical).

Nuclear staining assay. Apoptotic and necrotic cell death was analyzed by Hoechst33342 and propidium iodide (PI) co-staining. Cells were seeded at a density of 1×10^4 cells per well onto a 96-well plate and incubated overnight. After 24 h of ANG II treatment, the cells were washed and incubated with 10 μg/ml of Hoechst33342 and 5 μg/ml of PI for 30 min at 37˚C. Nuclei condensation and DNA fragmentation of apoptotic cells, as well as PI-positive necrotic cells, were visualized under a fluorescence microscope (Olympus IX51 with DP70 Digital camera system, Olympus, Central Valley, PA, USA).

Spheroid-formation assay. Spheroids were grown using a modified method from Kantara *et al*. (33). NCI-H460 cells were treated with ANG II for 5 days prior to spheroid-forming assay. Approximately 5×10^3 cells/well were seeded onto a 12-well ultralow-attachment plate using RPMI serum-free medium. After 7 days, primary spheroids were re-suspended into single cells, and again 5×10^3 cells/well were re-plated onto a 12-well ultralow-attachment plate using RPMI serum-free medium. Images of Secondary spheroids were captured and scored at day 7 under a phase-contrast microscope (Olympus).

In vitro 3D tumorigenesis assay. *In vitro* 3D tumorigenesis was determined on a matrigel-coated 96-well plate. A plate was coated with 0.5% matrigel which was left to solidify. NCI-H460 cells were suspended in culture medium containing 4% matrigel and different concentrations of ANG II. The cell suspension was seeded onto the coated plate at 300 cells/well. Culture medium containing ANG II was replaced every 2 days. After 7 days, colonies were visualized under microscope and colony size and number were evaluated using ImageJ software (NIH, Bethesda, MA, USA).

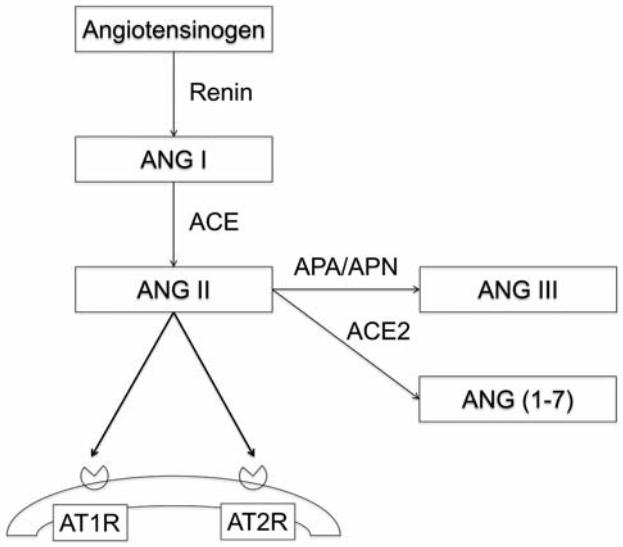

Figure 1. *The renin–angiotensin system. Angiotensinogen is a precursor of active angiotensin II (ANG II). Angiotensinogen is enzymatically cleaved by renin to generate a decapeptide angiotensin I (ANG I). Angiotensin I is inactive and subsequently cleaved by angiotensin-converting enzyme (ACE) to generate an octapeptide angiotensin II (ANG II). Angiotensin II can further cleave into angiotensin-(1-7) or angiotensin III by ACE2 and aminopeptidase A or N (APA/APN), respectively. ANG II exerts its biological effects through binding to two subtypes receptors belonging in G-protein-coupled receptor family, angiotensin II type 1 and type 2 receptors (AT1R, AT2R). Most physiological effects of ANG II are mediated by AT1R, whereas stimulation of AT2R results in counter-regulatory effects.*

Anchorage-independent growth assay. Anchorage-independent cell growth was evaluated by colony formation in soft agar. Soft agar was prepared using a 1:1 mixture of RPMI-1640 medium containing 10% FBS and 1% agarose. The mixture was allowed to solidify on a 24-well plate to form the bottom layer. Cells were then suspended in RPMI-1640 supplemented with 10% FBS and 0.33% agarose gel and seeded over the bottom layer at 3×10^3 cells per well. After the upper cellular layer was solidified, RPMI-1640 medium containing 10% FBS with ANG II was added to the system and incubated at 37˚C. The cells were fed every 2 days with 250 μl of complete medium. Colony formation was photographed under a microscope and relative colony number and size were determined.

Western blot analysis. After specific treatments, cells were incubated in a lysis buffer containing 20 mM Tris•HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 50 mM NaF, 100 mM Phenylmethylsulfonylfluoride, and protease inhibitor mixture from Roche Molecular Biochemicals (Indianapolis, IN, USA) for 1 h on ice. Cell lysates were analyzed for protein content using BCA protein assay kit from Pierce Biotechnology (Rockford, IL, USA). Equal amounts of denatured protein samples (40 μg) were loaded onto 10% SDS-polyacrylamide gel to undergo electrophoresis. The proteins were transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The transferred membranes were blocked with 5% non-fat dry milk in TBST (25 mMTris•HCl (pH 7.5), 125 mM NaCl, and 0.05% Tween 20) for 1 h prior to incubation with specific

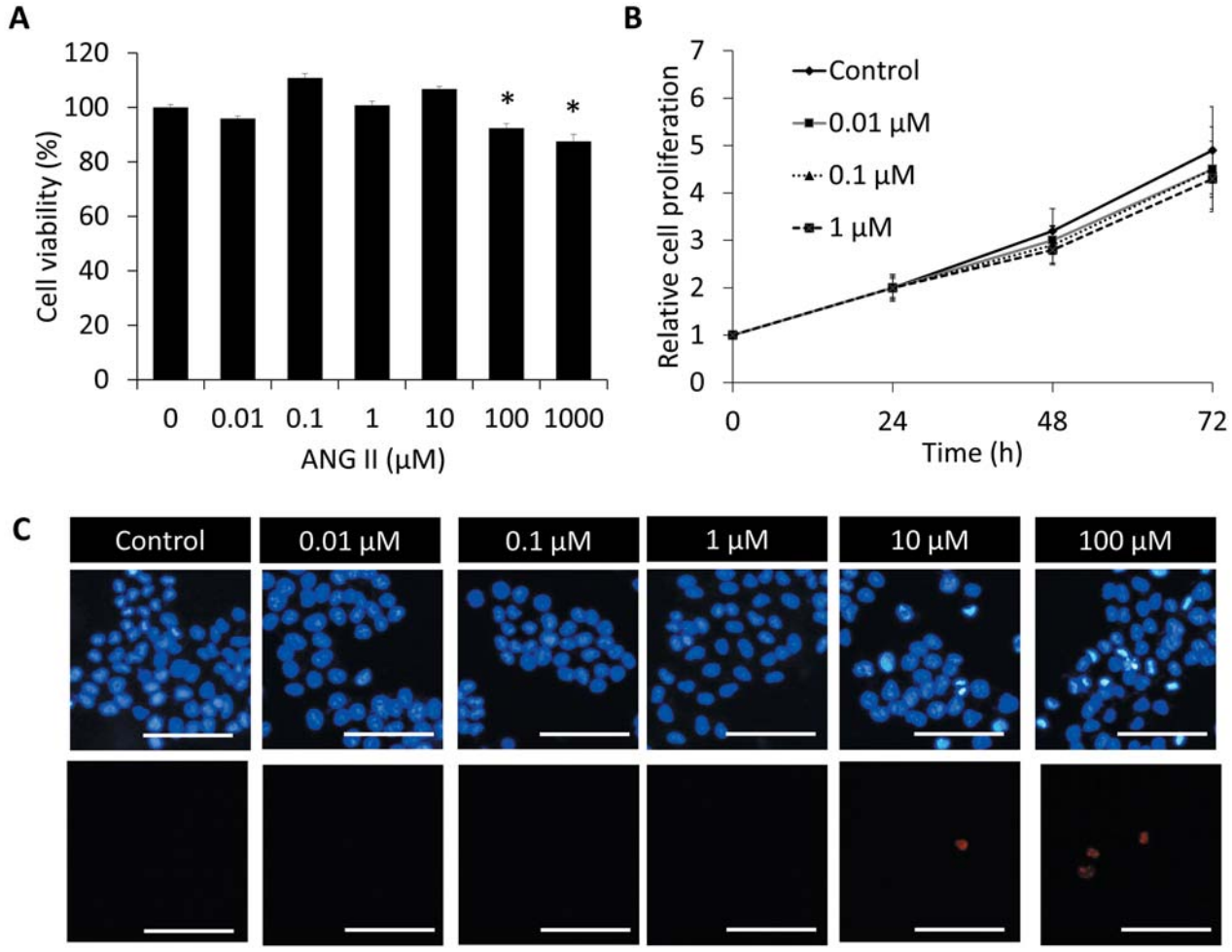

Figure 2. *Effects of angiotensin II (ANG II) on NCI-H460 non-small cell lung cancer cell line. A: The percentage of NCI-H460 cell viability. Cells were treated with different concentrations of ANG II (0.01-1000 μM) and cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay assay after 24 h. The viability of non-treated cells was taken as being 100%. B: NCI-H460 cell proliferation following exposure of the cells to ANG II (0-1 μM) for 24, 48, and 72 h. C: Hoechst33342/PI co-staining for nuclear morphology of the cells. Scale bars=100 μm. Results are expressed as fold increase in cell viability compared the control. Data are the mean±SEM (N=3). *p<0.05 vs. non-treated control.*

primary antibodies against CD133 (cluster of differentiation 133) (Cell Applications, San Diego, CA, USA), aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4˚C. The membranes were washed three times with TBST and incubated with the appropriate horseradish peroxidase-labeled secondary antibodies: anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA), anti-mouse IgG (Cell Signaling Technology), or anti-goat IgG (Santa Cruz Biotechnology), for 2 h at room temperature. The immune complexes were detected by SuperSignal West Pico Chemiluminescent substrate (Pierce Biotechnology) and exposed to film.

Statistical analysis. All treatment data were normalized to those for non-treated controls. Data are presented as the means±SD from three independent experiments. Statistical differences were determined using two-way ANOVA and a *post hoc* test at a significance level of $p<0.05$.

Results

Effects of ANG II on viability of H460 non-small cell lung cancer cells. Previous studies showed that ANG II induces apoptosis of human A549 adenocarcinoma alveolar epithelial cells in a dose-dependent manner (0-100 μM) (18, 19). However, it has been shown that at concentrations of 0-100 nM, ANG II increases the proliferation of human gastric cancer cells (20), suggesting that the concentration of ANG II and tumor heterogeneity may govern the effects of ANG II on

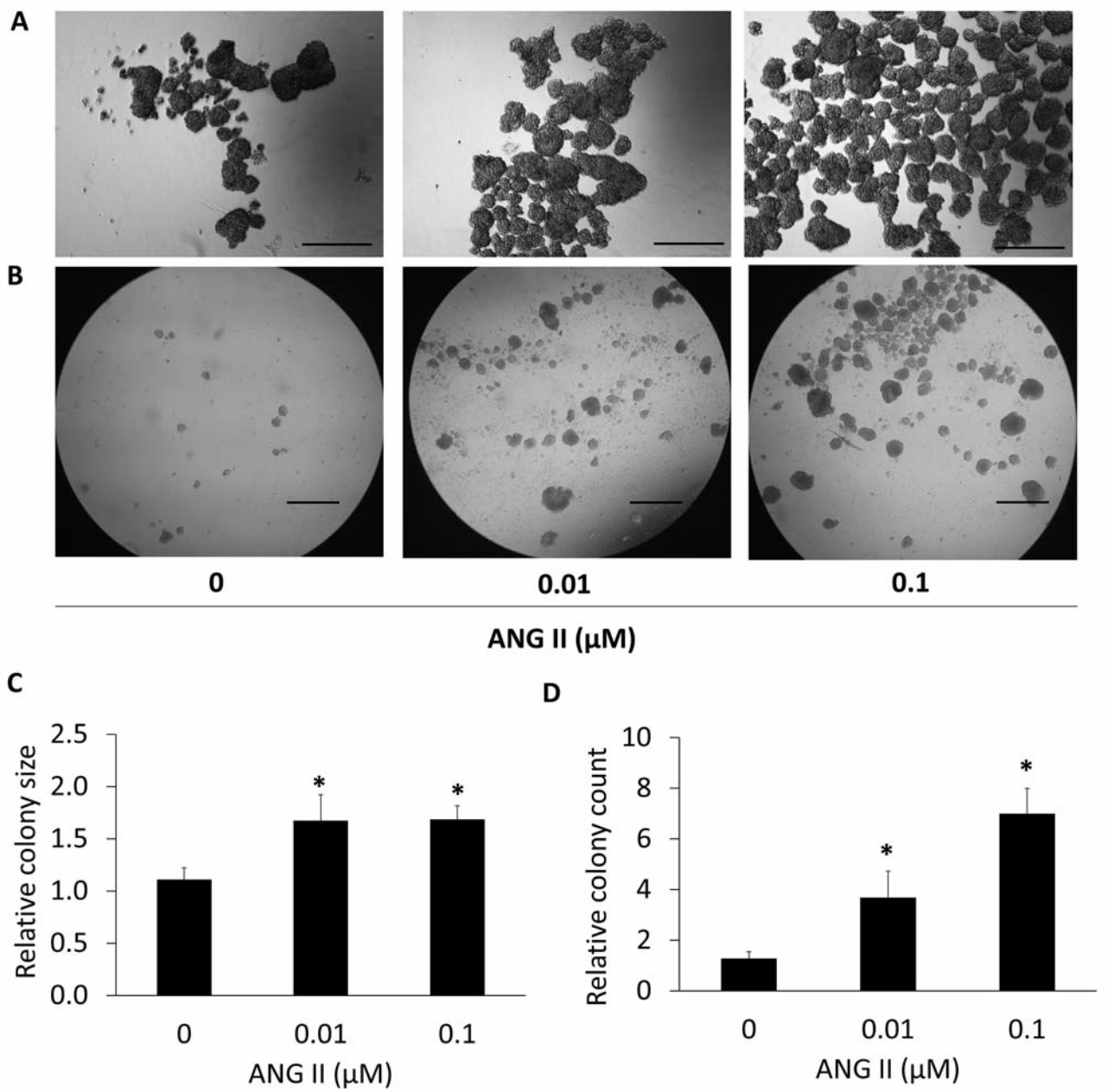

Figure 3. *Angiotensin II (ANG II) increases spheroid formation. NHI-H460 cells were pre-treated with ANG II (0-0.1 μM) for 5 days and subjected to sphere-formation assay. A: Representative phase-contrast images (×4 magnification; bar=500 μm) of secondary spheroid captured on day 7 for ANG II-treated and non-treated cells. B: Secondary spheroid clusters were gently separated with pipetting. Dissociated spheres were visualized under a microscope at ×4 magnification (bar=2500 μm). The images are representative from three independent experiments. The number (C) and size (D) of secondary spheroids were determined by the imageJ software on day 7 and expressed as the relative fold change compared to the control. Data are the mean±SEM of three independent experiments. *p<0.05 vs. non-treated control.*

cancer cell growth and proliferation. In order to study the effects of ANG II on CSC-like phenotype in H460 human lung cancer cells, we first determined cytotoxic and proliferative effects of ANG II on the cells. For the cytotoxicity assay, H460 cells were treated with serial dilutions of ANG II from 0 to 1000 μM for 24 h and cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. We found that treatment of the cells with ANG II at concentrations up to 10 μM had no significant cytotoxic effect on NCI-H460 cells. Cell viability began to significantly

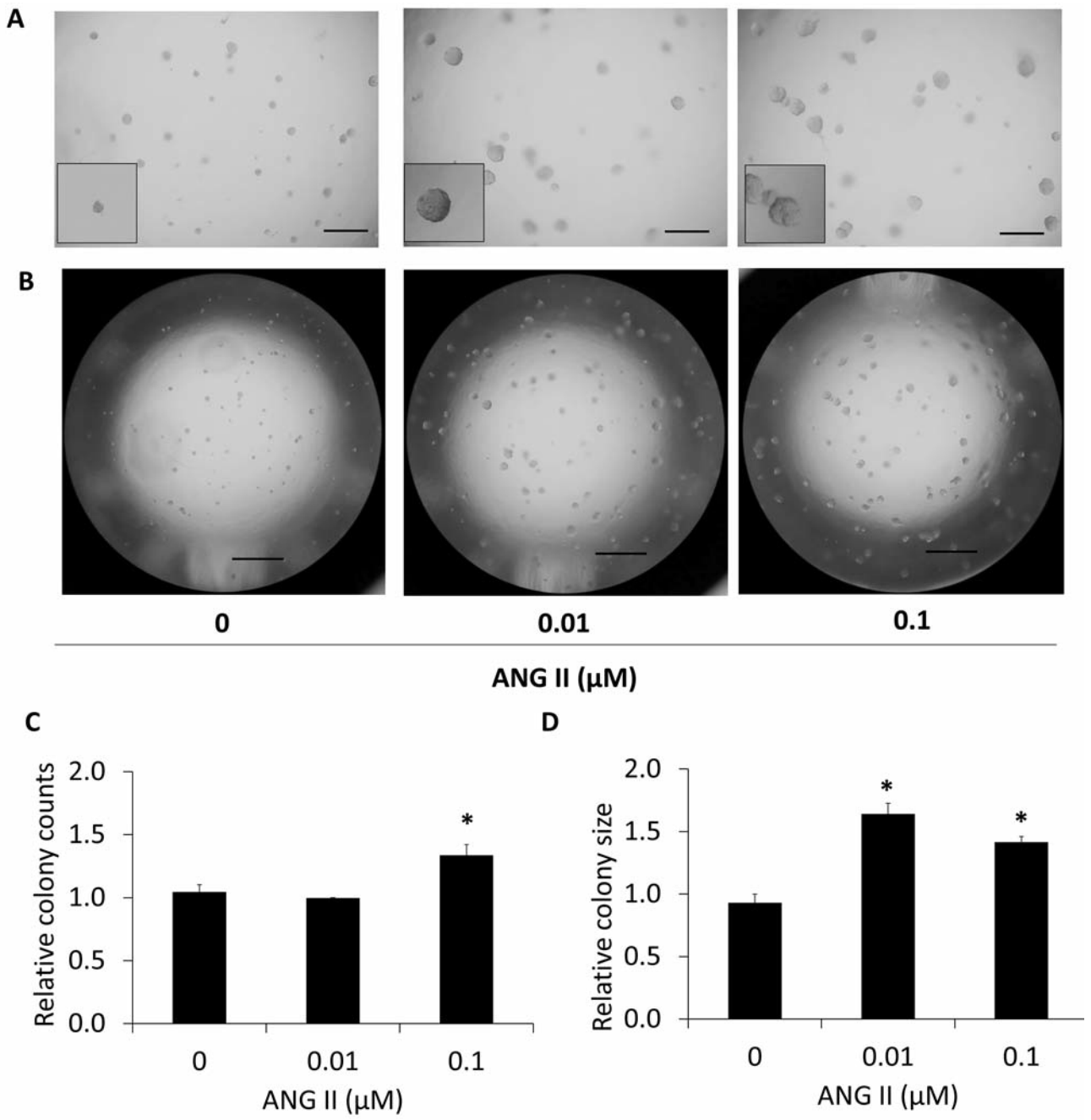

Figure 4. *Angiotensin II (ANG II) promotes tumor growth in a 3D tumorigenic assay. H460 cells were suspended in culture medium containing 4% matrigel and plated onto gel-coated 96-well plates. A: Images of colonies were obtained on day 7 at ×4 magnification (bar=2500 μm). Colony number (B) and size (C) in each field were analyzed and expressed as change relative to non-treated cells. Data are presented as the mean±SEM of three independent experiments. *p<0.05 vs. non-treated control.*

decrease in response to ANG II at 100 μM (Figure 2A). Hoechst33342/PI staining assay was performed to confirm the effect of ANG II on cell viability. The results indicated that condensed chromatin of apoptotic cells was initially observed in cells treated with 100 μM of ANG II (Figure 2C).

For effects on proliferation, NCI-H460 cells were treated with non-toxic doses of ANG II (0-1 μM) for 0-72 h and cell proliferation was determined using the MTT assay. Figure 2B shows that there was no significant alteration in terms of cell proliferation in all tested groups of cells. These data

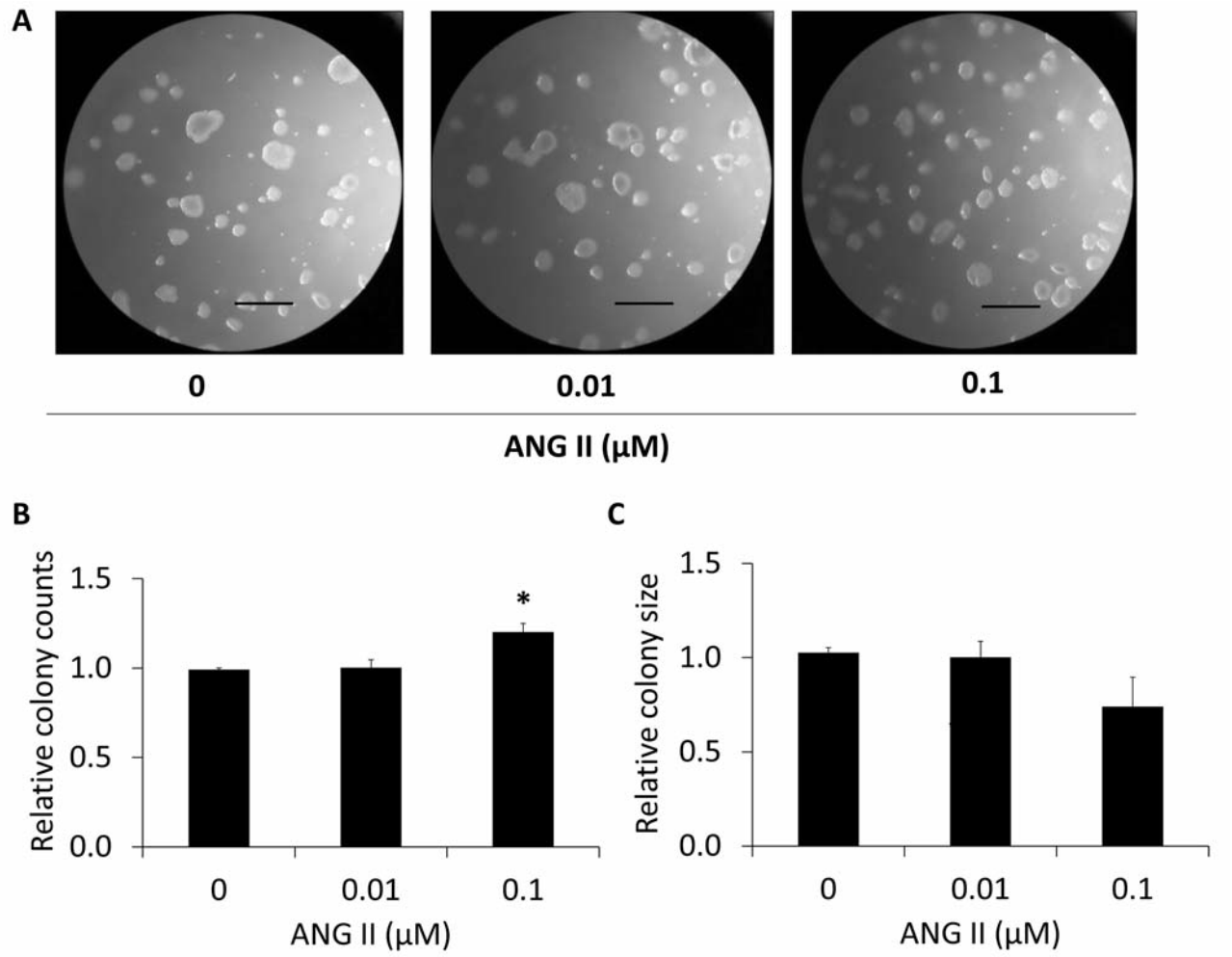

Figure 5. *Angiotensin II (ANG II) increases NHI-H460 cell survival in anchorage-independent conditions. NHI-H460 cells were treated with ANG II then subjected to soft agar colony formation assay. Representative fields were captured under a microscope at ×10 (A) and ×4 (B) magnification (bar=2500 μm). The number (C) and size (D) of colonies were evaluated relative to those of the non-treated control. Data are presented as the mean±SEM of three independent experiments. *p<0.05 vs. non-treated control.*

confirmed that ANG II concentrations of 0.01-0.1 μM did not have any cytotoxic or proliferative effects on H460 lung cancer cells.

ANG II increases spheroid formation. Two cardinal features of CSCs are their ability to undergo self-renewal and the ability to differentiate. The spheroid-forming assay has been widely used to evaluate these properties of stem cells. NCI-H460 cells were treated with ANG II for 5 days and subjected to spheroid-forming assay as described in Materials and Methods. Figure 3A shows representative phase-contrast images of secondary spheroids from ANG II-treated and control cells. To differentiate between cell aggregations through E-cadherin-mediated survival and dense spheroids proliferated from single colonies, floating sphere clusters were gently dissociated by pipetting and the remaining spheroids were evaluated under microscope (Figure 3B). Interestingly, we found that treatment with ANG II greatly increased the number and size of secondary spheroids in a dose-dependent manner in comparison to those of non-treated control cells (Figure 3C-D).

ANG II promotes tumor growth in 3D tumorigenic assay. Growing tumors in 3D cell culture has been proposed to mimic the tumor environment *in vivo* (21-23). We therefore sought to investigate whether ANG II promotes single-cell colony formation in the 3D assay. Figure 4A shows a representative 3D-matrigel colony. We found that 0.1 μM

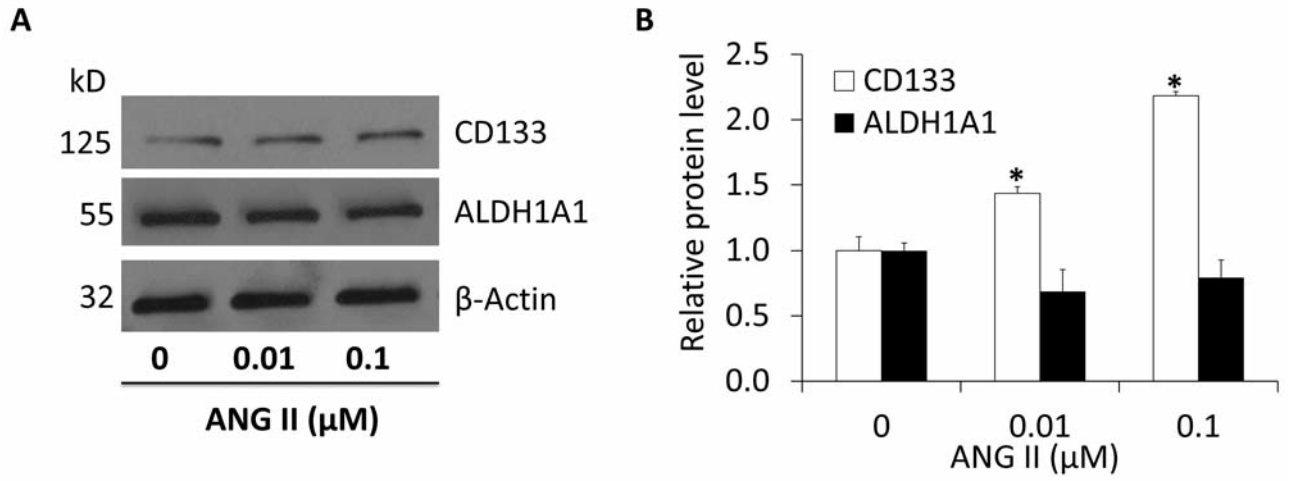

Figure 6. *Angiotensin II (ANG II) induces expression of cancer stem cell (CSC) markers. A: NHI-H460 cells were treated with ANG II (0–1.0 μM) for 5 days and analyzed for CSC markers CD133 (cluster of differentiation 133) and aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) by western blotting. β-Actin was used to normalize protein loading. B: Quantification of protein expression of CD133 and ALDHA1 normalized with β-actin relative to expression levels in non-treated cells. Data are the mean±SEM of four independent experiments. *p<0.05 vs. non-treated control.*

ANG II significantly increased the number of colonies compared to the non-treated group (Figure 4B). Treatment of the cells with ANG II also significantly increased colony size (Figure 4C). These results suggest that ANG II may potentiate the ability of cancer cells to survive and grow under 3D conditions.

ANG II increases NHI-H460 cell survival under anchorage-independent conditions. Having shown that ANG II could increase the ability of the cells to form tumor spheroids and grow in a 3D culture system, we next confirmed these results with an anchorage-independent growth assay. The ability of cancer cells to survive and grow in an anchorage-independent manner shows the capability of cancer cells to survive during metastasis. Anchorage-independent growth assay is also one of the recognized techniques for evaluation of CSC phenotype (2). Consistent with the results of the 3D-assay, ANG II at 0.1 μM significantly increased the number of colonies formed in soft agar compared to the control (Figure 5A). However, there was no significant difference in the colony size between cells treated with ANG II and the control group in this soft agar assay (Figure 5B). These results suggest that treatment of the cells with ANG II may influence cell survival under these conditions but not their growth rate. Representative images of colonies formed following ANG II treatments are shown in Figure 5C.

ANG II induces expression of CSC markers. To further investigate the effects of ANG II on CSC phenotype, we used two well-known CSC markers, namely CD133 and aldehyde dehydrogenase 1 family, member A1 (ALDH1A1). Figure 6A shows representative blots of CD133 and ALDH1A1 expression in NCI-H460 cells in response to ANG II treatment. Protein expressions are reported as the level relative to that of non-treated cells. Figure 6B indicates that ANG II significantly increased CD133 expression in a dose-dependent manner: 1.5- and 2.2-fold increases in CD133 expression were observed in response to 0.01 and 0.1 μM of ANG II, respectively. However, we found no significant increase in ALDH1A1 protein expression with ANG II treatment.

Discussion

There exists evidence suggesting a role of RAS in cancer progression, and that clinical treatment with RAS inhibition such as ACEIs and ARBs reduces cancer prevalence (24). We have shown, to our knowledge, for the first time that ANG II, a key modulator of RAS, has a possible role in regulation of CSC-like phenotype in human lung cancer cells. The promotory effect of ANG II on CSC phenotype, including on survival and growth under detached conditions, and on the ability to form tumor spheroids has been demonstrated in the present study. In addition, the well-known CSC marker CD133 was clearly induced in these lung cancer cells following ANG II exposure (Figure 6).

Having been identified in solid tumors, CSCs are generally acknowledged to be a basis for tumor initiation, development, metastasis and relapse after therapy. A rare population of CSCs has been identified as being immortal tumor-initiating cells with self-renewal and pluripotent capacity (25). Small numbers of CSCs generate unlimited

progeny of differentiated tumors in various animal models of cancer (26-28). More importantly, previous studies have shown that metastases can directly arise from CSCs (29), possibly due to the association between the EMT, a critical event in cancer metastases, and CSCs acquisition (30).

Previous studies have reported local expression of RAS components in various cancer cells and tissues (12, 20, 31-33) and dysregulation of this network correlates with cancer invasiveness and metastasis in clinical studies (34). In non-small cell lung cancer, patients receiving either ACEIs or ARBs had longer median survival than non-recipients (35). Administration of captopril or candesartan, a common ACEI and ARB respectively, reduced tumor volume and lymph node metastasis in non-small cell lung cancer xenografts (10). In addition, ANG II activates cell proliferation in prostate cancer and oral administration of ARB to nude mice reduced growth of transplanted tumors in a dose-dependent manner (12).

ANG II mediates its physiological actions mainly through two receptor sub-types including AT1R and AT2R. AT1R, a G-protein-coupled receptor, also couples to various tyrosine kinase including those of the mitogen-activated protein kinase family and the Janus kinase/Signal transducers and activators of transduction signaling (36). AT1R tyrosine kinase activation is thought to be a signaling mechanism behind ANG II-induced cell hypertrophy and proliferation of cancer cells (4). However, activation of AT2R signaling induces apoptosis in adenocarcinoma lung cancer (37), emphasizing the opposing roles of the two sub-types. Transactivation of epidermal growth factor receptor (EGFR) is also another important mechanism linking AT1R to tyrosine kinase pathways (38-40). A recent study revealed that chronic exposure to ANG II induces EMT in renal epithelial cells through transactivation of EGFR. In that study, AT1R activation led to the production of reactive oxygen species and activation of Src kinase, thereby resulting in phosphorylation of scaffold protein caveolin-1 and prolonged EGFR signaling activation, which may alter gene expression and a phenotypic change to EMT (41). Besides, in intrahepatic cholangiocarcinoma, ANG II enhanced the expression of EMT markers and migration capability of cultured cells (15).

Although the significant roles of ANG II in tumor growth and metastasis have been defined, the connection between ANG II and CSC-like phenotype has not been elucidated. In the present study, we showed for the first time that ANG II induces CSC marker expression and promotes cancer aggressiveness in human non-small cell lung cancer cells. This study supports a role of endogenous ANG II in cancer aggressiveness. Further studies in molecular mechanisms, as well as the *in vivo* effects, will be useful towards future use of conventional drugs that regulate RAS, such as ACEIs or ARBs in cancer treatment or prophylaxis.

Acknowledgements

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